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14. ABSTRACT

A summary is presented of research performed during three years of a project to determine the role of Cdc25 phosphatases in human breast cancer. Three specific aims were pursued. The first was to determine the role of Cdc25B in breast cancer proliferation. The second aim was examining whether alternative splicing of Cdc25C contributes to human breast cancer. The final aim was to explore a potential novel breast cancer therapy involving altered expression of Cdc25C. The long term goals of this research were to validate a clear role for Cdc25B in breast tumor cell proliferation and to rigorously determine whether Cdc25C may contribute to human breast tumorigenesis in other ways besides its overexpression.

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Introduction

Cell cycle progression is mediated by cyclin-dependent kinases. These kinases are, in turn, regulated by their own phosphorylation. The family of Cdc25 phosphatases is responsible for removing inhibitory phosphates from these kinases thereby triggering advancement through the cell cycle (Boutros et al., 2007). There are three members of this family. Two members of this family, Cdc25B and Cdc25C function in a similar manner and have been implicated in regulating entry into mitosis via removal of inhibitory phosphorylations on the Cdc2 kinase (Draetta and Eckstein, 1997). Interestingly, only Cdc25B has been clearly shown to play a role in human cancer, although the precise mechanism remains unclear (Kristjansdottir and Rudolph, 2004; Rudolph, 2007). Nevertheless, Cdc25C has not been rigorously excluded as contributing to human cancer. It was hypothesized that both Cdc25B and Cdc25C contribute to human breast tumor development. Thus, the focus of this research was to validate a role for Cdc25B in regulating breast tumor cell proliferation and to explore other ways that Cdc25C may contribute to the oncogenic phenotype beside overexpression. With this in mind, three specific aims were pursued. Cdc25B has been reported to be overexpressed in a variety of human tumor types. In the first aim, the levels of expression of Cdc25B were examined in human breast tumor cell lines. Levels of expression of Cdc25B were manipulated in these cell lines to determine effects on proliferation. Cell culture models will be employed to examine the role of Cdc25B in transformation. A role for overexpression of Cdc25C in human cancer has not been found in previous studies. It was therefore hypothesized that there are other mechanisms involving Cdc25C, one of which may be regulation by alternative splicing. In the second aim, the various splice forms of Cdc25C were to be characterized and their role in regulating cell growth were to be examined. In addition, breast tumor cell lines were to be screened for alterations in Cdc25C splicing. Overexpression of Cdc25C has been shown to sensitize cells to DNA damaging agents. p53 downregulates Cdc25C expression in response to such agents, and this repression requires the cooperation of another cellular factor (St. Clair et al., 2004). It is suggested that by abrogating the ability of p53 to repress Cdc25C expression in response to particular chemotherapeutic agents, it will be possible to induce a cytotoxic response. It was hypothesized that by blocking the expression of the cooperating factor, such a therapeutically desirable outcome may be achieved. The focus of this aim was to identify and characterize such a factor as well as perform “proof of principle” studies to validate this overall approach. The studies reported here summarize the research performed during a three-year funding period.

Body

Task 1. Screen breast tumor cell lines for levels of cdc25B and cdc25C expression (Months 1-4)

Eight breast tumor cell lines were chosen for study in comparison to the non-transformed but immortalized MCF10A breast epithelial cells. Three of these tumor lines express a wild-type p53 while the remaining nine have sustained missense mutations as shown in Table 1. Protein extracts were prepared from multiple breast tumor cell lines and immunoblotting analysis was performed (Figure 1). Levels of p53 expression correlated with the known p53 status of these cells. Thus, cell lines without p53 mutation expressed low levels of the protein whereas the mutant p53 was expressed, as expected, at substantially higher levels. The basal level of Cdc25C varied, but also correlated with p53 status. Thus, cells with wild-typ53 p53 expressed low levels of Cdc25C whereas those that had sustained p53 mutation expressed high amounts of Cdc25C. This is consistent with previous studies from out laboratory that p53 transcriptionally represses Cdc25C gene expression (St. Clair et al., 2004). This data argues however that p53 status is the major determinant for Cdc25C protein expression. Mdm2 is a negative regulator of p53 that has been shown to, in turn, be transcriptionally upregulated by p53. Examination of levels of Mdm2 expression in these various cell lines showed that in contrast to Cdc25C, there was no strong correlation between Mdm2 expression and p53 status (Figure 1). However, our findings are consistent with a recently published study showing that a single nucleotide polymorphism in the Mdm2 gene (SNP309) was an important determinant of Mdm2 levels in breast cells that also express estrogen receptor (Hu et al., 2007). Thus, T47D and MCF7 cells show high levels of Mdm2 expression while ZR-75-1 cells have reduced expression (Figure 1), correlating with the SNP309 status in these cells (Table 1). Taken together, this argues that levels of Cdc25C expression, in contrast to another p53 target, Mdm2, is strictly determined by p53 status. The levels of Cdc25B is consistently higher in all tumor lines as compared to the non-transformed MCF10A cells and this is independent of p53 status (Figure 1). Thus, these eight tumor lines are suitable for further study of the role of Cdc25B overexpression in their proliferation and tumorigenic properties. Examination of transcript levels using an RT-PCR approach shows that protein levels reflect amounts of Cdc25C and Cdc25B messenger RNA in these cells (Data not shown).

These findings support the idea that Cdc25B is overexpressed in human breast cancer and is likely to be an important determinant of tumorgenicity. Higher levels of Cdc25C that are found in mutant p53-expressing tumor lines may also contribute to the neoplastic properties of those cells and needs to be further explored.

Table 1. Characteristics of breast cell lines

	MCF7	MDA-MB-231	MDA-MB-361	MDA-MB-468	ZR-75-1	SKBr3	T47D
p53	wild-type	R280K	wild-type	R273H	wild-type	R175H	L194F
Estrogen receptor	+	-	+	-	+	-	+
Progesterone receptor	+	-	+	-	+	-	+
Mdm2 SNP309	T/G				T/T		G/G

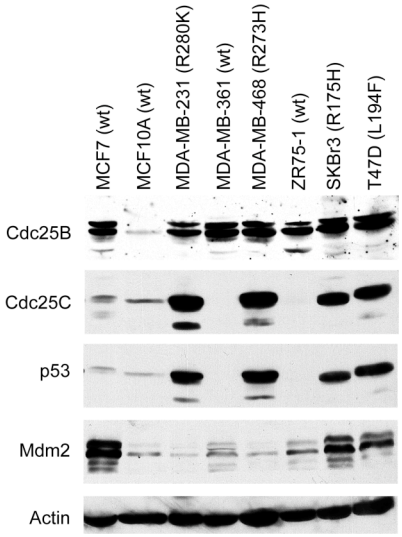


Figure 1. Expression levels of Cdc25B and Cdc25C in multiple breast tumor cell lines.
The indicated cell lines were lysed and subjected to immunoblotting for the indicated proteins. Actin is included as a loading control.

Task 2. Abrogate cdc25B expression in overexpressing breast tumor cell lines (Months 5-12)

To determine the relevance of high levels of Cdc25B expression for the proliferation of breast tumor cells, an siRNA approach was designed to ablate expression of Cdc25B. As the levels of Cdc25C were also found to be high in a subset of the breast cancer lines that express mutant p53 (Figure 1), a strategy for downregulation of Cdc25C was also attempted. The siRNA approaches to ablate expression of either Cdc25B or Cdc25C were successful as determined by immunoblotting for protein expression (Figure 2). Interestingly, loss of Cdc25B expression resulted in an alteration in the cell cycle profile of these cells whereas downregulation of Cdc25C had no effect (Figure 2). Although it was gratifying that both siRNA strategies were successful, it was found that loss of Cdc25B expression affected cell proliferation regardless of their transformed phenotype (Data not shown). This suggests that under these conditions it has an important role in regulating normal cell cycle progression. As ablation of Cdc25B affects cell proliferation regardless of their initial levels of Cdc25B

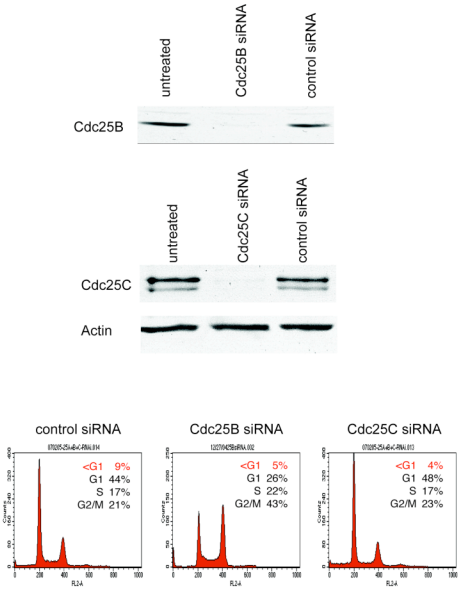


Figure 2. Ablation of Cdc25B but not Cdc25C expression affects cell cycle progression.
MCF10A cells were transfected with either control siRNA oligonucleotides or those directed towards either Cdc25B or Cdc25C as indicated. Cell lysates were then immunoblotted for the indicated proteins. Corresponding dishes of cells were subjected to propidium iodide staining and flow cytometric analysis. Percent of cells with the indicated DNA content were then calculated.

expression, the approaches that were optimized here will be uninformative for determining the significance of elevated Cdc25B in the growth of the breast cancer cell lines identified in Task 1. Methods to reduce Cdc25B levels to that seen in MCF10A cells rather than complete ablation of Cdc25B expression was considered to be one way to address this. Attempts to achieve to a partial downregulation of Cdc25B proved to be difficult, as it requires a method to carefully titrate the siRNA oligonucleotides and this was unfortunately not achieved. The effects of overexpression of Cdc25B were then examined as a complement to the downregulation studies proposed in the original application.

Preliminary studies were performed to examine the effect of overexpression of wild-type Cdc25B in wild-type p53-expressing U2OS cells Surprisingly, this caused premature entry into mitosis and cell cycle arrest. This was dependent upon the phosphatase activity of Cdc25B, as a catalytically dead mutant failed to exert these effects. p53 was induced and phosphorylated on ser15 but not on ser315. Increased phosphorylation of ATM as well as chk1 and chk2 was seen, implicating the ATM/ATR pathways in this response. The p53 target, the cyclin-dependent kinase inhibitor p21, was also upregulated in a manner requiring p53. Downregulation of either p53 or p21 using siRNA eliminated the cell cycle arrest. While 40% of cells showed detectable immunostaining for γ-H2AX, elimination of p53 caused 100% of the cells to stain positive. Consistent with these results, ectopic expression of Cdc25B inhibited long term growth in several different cell lines. As these effects are seen in cells that are null for $p14^{ARF}$, signaling via $p14^{ARF}$ is not involved. These results show that premature entry into mitosis by overexpression of Cdc25B triggers DNA damage checkpoint responses, most likely through hyperactivation of cyclin-dependent kinases. It is proposed that inappropriate expression of Cdc25B transmits two distinct signals to p53. One stems from activation of DNA-damage pathways including ATM and ATR. The other is distinct from those pathways given that it may occur cells that are γ-H2AX-negative. These results further highlight the importance of p53 in modulating the cellular response to deregulated proliferation.

We then compared ectopic expression of Cdc25B with that of Cdc25C and found that Cdc25B but not Cdc25C inhibits cell proliferation in long-term assays. Chimeric proteins generated from the two phosphatases show that the anti-proliferative activity is associated with the C-terminal end of Cdc25B. Indeed, the catalytic domain of Cdc25B is sufficient to suppress cell viability, in a manner dependent upon its C-terminal 26 amino acids. This region of Cdc25B is shown to be critical for substrate recognition and affinity. These results demonstrate key differences in the biological activities of Cdc25B and Cdc25C due to differential substrate affinity and recognition. This also argues that the antiproliferative activity of Cdc25B needs to be overcome in order for it to act as an oncogene during tumorigenesis. Although these studies were not planned as part of the original statement of work and fall outside of the plans in the research application the findings are intriguing and have led to one accepted publication (Varmeh-Ziaie and Manfredi, 2007) and a second that is under revision (Varmeh-Ziaie and Manfredi, in revision). The U2OS (osteosarcoma) and HCT116 (colon carcinoma) cell lines were used initially as they have been well characterized in terms of cell cycle responses and are technically amenable to the approaches used here. Despite some technical challenges, these results were then validated in wild-type p53 expressing MCF-7 breast cells.

Task 3. Compare ability of cdc25B and cdc25C to cooperate with activated ras in transformation of mammary epithelial cells (Months 13-26)

In order to examine the role of the N-terminal ends of Cdc25B and Cdc25C in their activity, it was proposed to make hybrid proteins. To this end, chimeras in which the N-termini of Cdc25B and Cdc25C were exchanged were generated (25B/C and 25C/B) (Figure 3). 25B/C consists of residues 1-390 of Cdc25B fused to residues 281-473 of Cdc25C (Figure 3). The N-terminus of 25C/B encompasses residues 1-280 of Cdc25C and it was fused to residues 391-580 of Cdc25B (Figure 3). The chimeras were generated by blunt-end cloning to avoid possible influence of irrelevant sequences. As controls, constructs expressing only the catalytic domains of Cdc25B (ΔN25B) and Cdc25C (ΔN25C) and their corresponding catalytically inactive mutants were generated (Fig. 3). ΔN25B contains residues 391-580 of Cdc25B and ΔN25C residues 281-473 of Cdc25C. All constructs were FLAG-tagged at the N-terminus. Each of these constructs or empty vector was cotransfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours post-transfection, cell samples were lysed and immunoblotted with FLAG antibody to verify similar expression levels and size of the proteins (Figure 4). To further validate these constructs, the immunoblots were then stripped and subsequently probed with specific antibodies against the N-terminus or C-terminus of Cdc25B or Cdc25C (Figure 4). These constructs will be used in the planned studies in MCF-10A cells to examine their transforming activity.

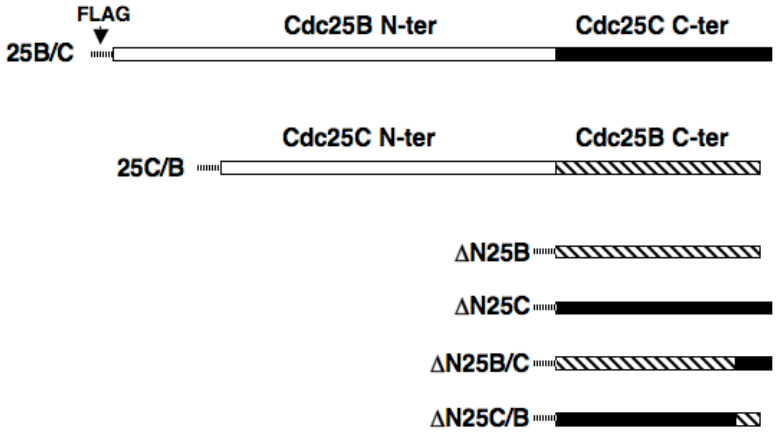


Figure 3. Expression constructs for FLAG-tagged chimeras of Cdc25B and Cdc25C and their isolated C-terminal regions were generated.
Sequences corresponding to the N- and C- terminal ends of Cdc25B and Cdc25C were PCR amplified. The N-terminal end of Cdc25B encompasses residues 1-390 and that of Cdc25C residues 1-280. The C-terminal end of Cdc25B (shaded) and Cdc25C (black) consists of residues 391-580 and residues 281-473 respectively. The constructs expressing 25B/C and 25C/B were generated by blunt-end ligation of the indicated parts of the proteins. ΔN25B and ΔN25C each expresses the C-terminal ends of Cdc25B and Cdc25C respectively. The constructs expressing ΔN25B/C and ΔN25C/B were generated by fusion PCR. ΔN25B/C expresses residues 391-554 of Cdc25B fused to residues 445-473 of Cdc25C. ΔN25C/B expresses residues 281-444 of Cdc25C fused to residues 555-580 of Cdc25B. All the constructs were FLAG tagged at their N-terminal ends. The position of the FLAG epitope is indicated.

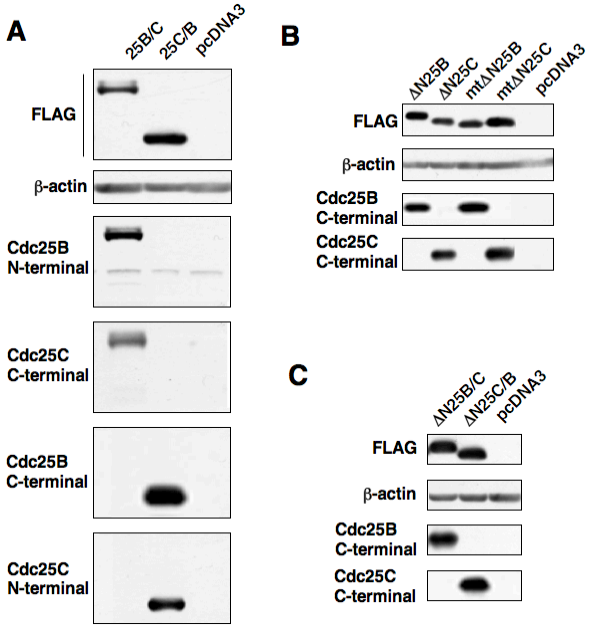


Figure 4. Various Cdc25B and Cdc25C proteins are expressed at comparable levels.
pcDNA3 vectors expressing the indicated FLAG-tagged proteins or empty vector were transfected into cells. Twenty-four hours later cells were lysed and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody or specific antibodies against the N- or C-terminus of Cdc25B or Cdc25C. β-actin is loading control. (A) 25B/C and 25C/B chimeras. (B) ΔN25B and ΔN25C and their corresponding catalytically inactive mutants (C) ΔN25B/C and ΔN25C/B.

It was proposed to then determine whether overexpression of Cdc25B or Cdc25C was capable of transforming primary cells and to examine the different constructs in these assays. Although it was originally planned to use a mouse cell line C127I, a recent publication highlighted the utility of the non-transformed human breast line MCF-10A for such assays. Importantly, it was shown that a third member of the Cdc25 family, Cdc25A was required for transformation of MCF-10A cells by activated Ras and a dominant-negative p53 (Ray et al., 2007). With this in mind, attempts were made to establish conditions to perform transformation assays using these human cells. While the use of human cells for these studies would have provided findings that are much more relevant to the goal of determining the role of Cdc25 family members in human breast tumorigenesis, these proved to be unsuccessful.

Task 4. Screen breast tumor cell lines for alternatively spliced forms of cdc25C (Months 1-6)
Preliminary data was presented within the grant application to show that MCF7 and ZR-75-1 cells express two isoforms of Cdc25C. It was thus proposed that alterations in Cdc25C isoforms expression may contribute to breast tumorigenesis. Additional analysis has shown that the relative expression pattern of these two isoforms is unaffected by the tumorigenic phenotype of the cells being examined. Thus, non-transformed MCF-10A cells have a similar expression pattern as the breast tumor lines (Data not shown). These findings do not support one of the hypotheses being tested here, namely that alterations in the relative expression of various Cdc25C isoforms contributes to breast cell transformation. Thus, enthusiasm for further characterization of individual splice forms was reduced and not pursued.

Task 5. Determine the role of individual splice forms of cdc25C (Months 10-30)
It was decided not to pursue this task because of the findings obtained in Task 4 (see above).

Task 6. Confirm effect of cdc25C overexpression in human breast tumor cells (Months 16-36)

Downregulation of Cdc25C by p53 or its phosphorylation by Chk1 and subsequent cytoplasmic sequestration has been shown to contribute to the G2 arrest caused by genotoxic stress (St Clair et al., 2004). To confirm this effect in breast epithelial cells, MCF-10A cells were infected a recombinant adenovirus expressing Cdc25C (Ad-25C) 24 hrs prior to treatment with doxorubicin or empty vector with the same multiplicity of infection (Figure 5). At this time point more than 95% of cells were infected as judged by microscopic evaluation of green-fluorescent protein (GFP) expression. Uninfected cells were included as control. The cell-cycle profile of these cells was examined 48 hrs after drug treatment by flow-cytometry analysis (Figure 5). Parental MCF-10A cells or those infected with empty adenovirus were able to arrest with a 4N DNA content representing a G2 arrest (Figure 5). MCF-10A cells overexpressing Cdc25C, however, were unable to maintain the G2 arrest and undergo apoptosis as evidenced by an increase in cells with a hypodiploid DNA content (Figure 5).

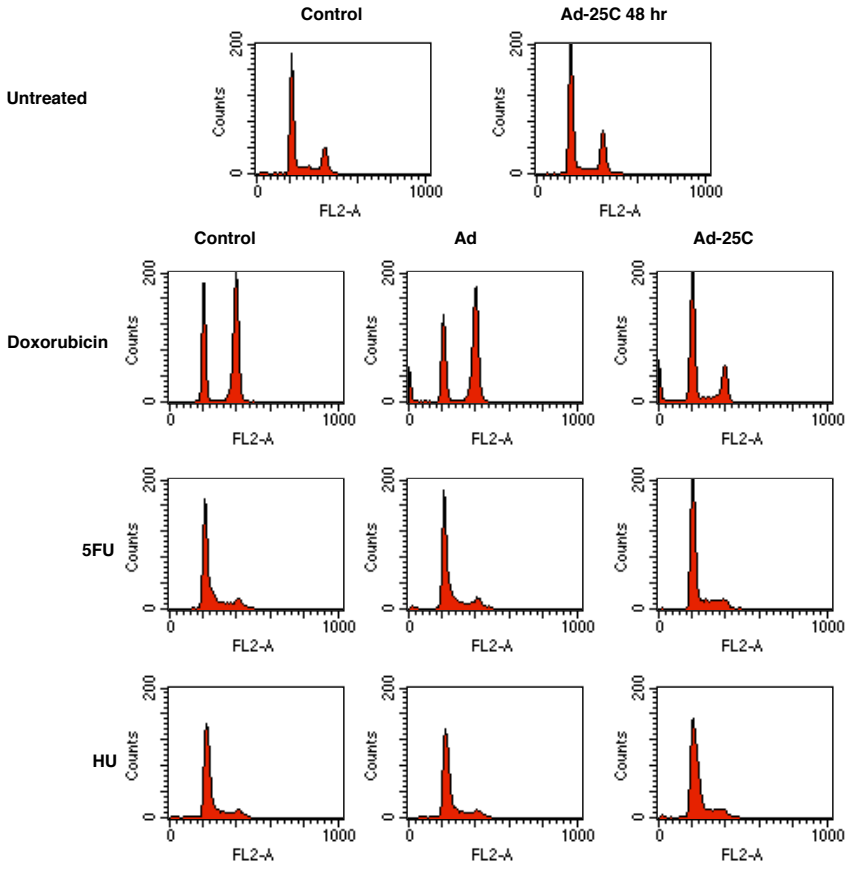


Figure 5. Adenovirus-mediated overexpression of Cdc25C abrogates the cell cycle arrest and sensitizes breast cells to doxorubicin, but does not affect the cellular response to 5-fluorouracil or hydroxyurea.
MCF-10A cells were infected with the indicated adenoviruses for 24 hr and then treated with doxorubicin, 5-fluorouracil, or hydroxyurea for 48 hr. Cells were stained with propidium iodide prior to flow-cytometry analyses.

In order to investigate the effect of excess amount of Cdc25C on cell-cycle arrest in G1/S or S-phase of the cell cycle, Cdc25C-overexpressing cells were treated with either of two chemotherapeutic agents, 5-fluorouracil (5-FU, 50 μM), an inhibitor of pyrimidine synthesis, or hydroxyurea (HU, 2mM), an inhibitor of both purine and pyrimidine synthesis. Cell-cycle profiles of these cells at the indicated time points were measured by flow-cytometry analyses (Figure 5). These results indicate that excess Cdc25C only overrides the G2 arrest caused by doxorubicin and has no effect on the G1/S arrest resulting from 5-FU and HU. These findings confirm that the ability of Cdc25C to sensitize cells to doxorubicin occurs in breast epithelial cells.

Task 7. Perform "proof of principle" studies (Months 8-24)

Four cdc25C promoter-driven expression plasmids have been generated and are illustrated schematically in Figure 6. Previous studies have shown that enforced expression of Cdc25c abrogates the p53-dependent checkpoints in response to DNA damaging chemotherapy (St. Clair et al., 2004). It was hypothesized that if the p53-dependent repression of Cdc25C could be ablated, that the cellular response could be similarly converted from a cell cycle arrest to an apoptotic outcome. As a "proof of principle" it was proposed to drive expression of Cdc25C in breast tumor cells in a manner independent of p53. The long-term goal is to identify the factor that interacts with the GC box, inhibit its activity, and thereby prevent Cdc25C downregulation. To

determine the feasibility of this approach before the factor is actually identified (the goal of Task 8), promoters that drive Cdc25C expression that lack the binding site for this putative factor have been generated (ΔGC). During the design of this initial construct, it was realized that p53-dependent repression via the CDE/CHR element may make interpretation of the results difficult. Thus, the activity of this element was also ablated in a matched set of constructs by scrambling four bases within the CHR.

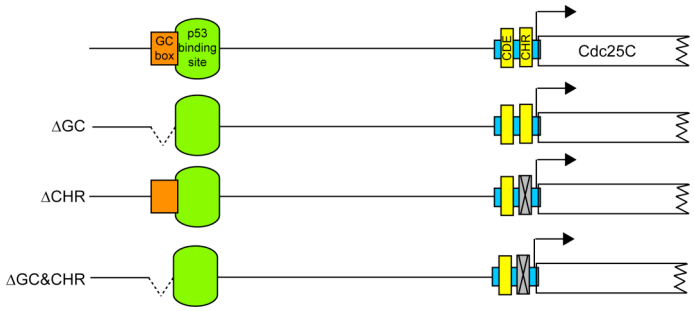


Figure 6. Generation of expression plasmids that drive expression of Cdc25C under control of the human Cdc25C promoter.
The sequence of the human Cdc25C promoter from -225 to +1 was inserted upstream of a cDNA expressing human Cdc25C. Three derivative of this plasmid were then generated. In ΔGC, the eight bases upstream of the p53 binding site (-163/-156) were deleted. In ΔCHR, four bases within the CHR element were scrambled. In ΔGC&CHR, both of these alterations were incorporated in the same construct.

Previous studies have shown that DNA damage-induced downregulation of Cdc25C occurs in a p53-dependent manner (St. Clair et al., 2004). To confirm that this effect occurs in breast tumor cells, the MCF7 cell line was treated with doxorubicin. Induction of p53 protein and its target the cyclin-dependent kinase inhibitor p21 was observed. The levels of Cdc25C protein as well as that of the cell cycle regulators Cdc2 and Cyclin B1 were also decreased (Figure 7, left). RT-PCR analysis confirmed the downregulation of Cdc25C also occurred at the mRNA level (Figure 7, right). To explore the p21-dependence of this, an siRNA approach was used to ablate p21 expression. Downregulation of p21 abrogated the decrease in Cdc25C, Cdc2, and Cyclin B1 protein levels as well as that of Cdc25C messenger RNA (Figure 7). These results confirm and expand the published findings in cell lines from other tumor types. Thus, MCF7 cells are validated as a suitable cell line to perform the "proof of principle" studies. Constructs were generated to restore Cdc25C expression to cells in which Cdc25C had been downregulated by an siRNA approach. The establishment of clones using MCF7 cells was attempted but it became apparent that comparisons between different clones will be problematic if they contain differing copy numbers of the transfected plasmids.

Task 8. Identify factor involved in p53-dependent repression (Months 1-36)

To screen candidate factors for a role in p53-dependent repression, we have utilized an siRNA approach. Sp1 and Sp3 expression have been ablated individually using transient transfection with siRNA oligonucleotides which effectively downregulate their levels. Neither of these showed any effect on p53-dependent repression of cdc25C (Figure 8). We have had difficulty obtaining suitable immunological reagents to use to detect other candidate factors (notably members of the KLF family of transcription factors). Although siRNA oligonucleotides are available that have been reported to affect expression of several of these (KLF4, KLF5, and KLF6), until we can establish a means to detect expression of endogenous proteins, we are unable to address their role in repression by p53. Thus, we have begun to screen a battery of commercially available antibodies to see which will be useful. We have also come to realize that this candidate approach may be problematic if there is redundancy among GC box-binding proteins in our list of likely candidates. There are four Sp family members and 12 in the KLF family (Turner and Crossley, 1999). Although ablation of each of these individually is feasible and will be pursued, the possibility of performing combinatorial knock-down of two at a time, for example, may become technically difficult. Given these concerns, we are now focusing on the proposed biochemical purification approach to identify novel factors with the hope of validation using an siRNA approach. To facilitate this analysis, the minimal element that is involved in p53-dependent repression needed to be identified. The previous studies had shown that three 10 bp p53 binding repeats were present. Deletion analysis has now shown that loss of the last 10 bp does not affect repression (Figure 9). In the context of this element (-163/-136), mutation of two bases within the adjacent GC box to either TT or AA abrogates repression as was reported for the larger sequence (Figure 5). We now feel that we have identified a minimal element for further study in the biochemical assays proposed.

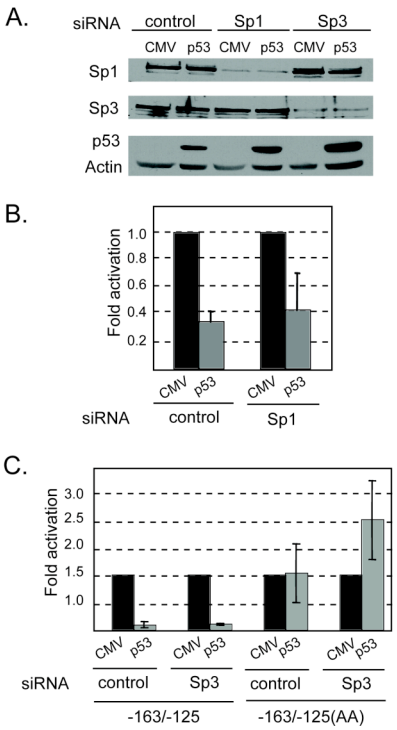


Figure 8. Ablation of either Sp1 or Sp3 does not affect p53-dependent repression of the Cdc25C element.

(A) Cells were transfected with either control siRNA oligonucleotides or those directed against Sp1 or Sp3. Cell lysates were prepared and immunoblotted for the indicated proteins. (B-C) Corresponding dishes of cells were co-transfected with either vector alone (CMV) or an expression plasmid for p53 as well as the indicated luciferase reporters. Fold activation was determined compared to the values obtained for each reporter in the presence of vector. Data represents the average of three independent experiments performed in duplicate. Error bars represent standard deviation.

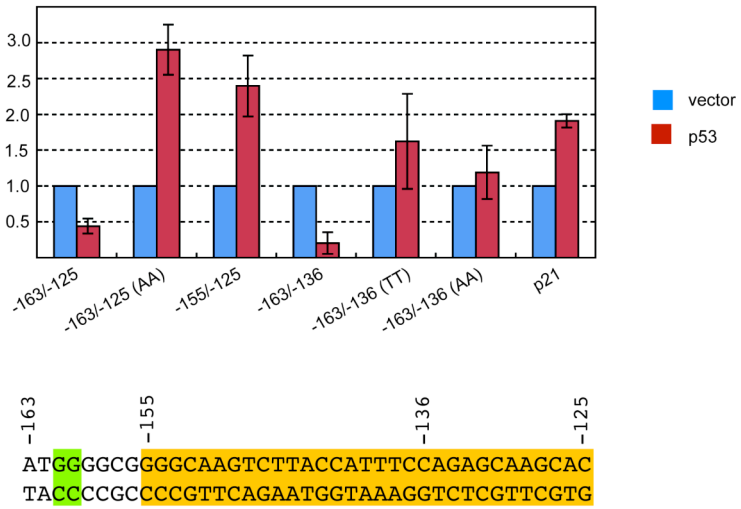


Figure 9. The minimal p53 responsive element for transcriptional downregulation of Cdc25C is contained within -163/-136.

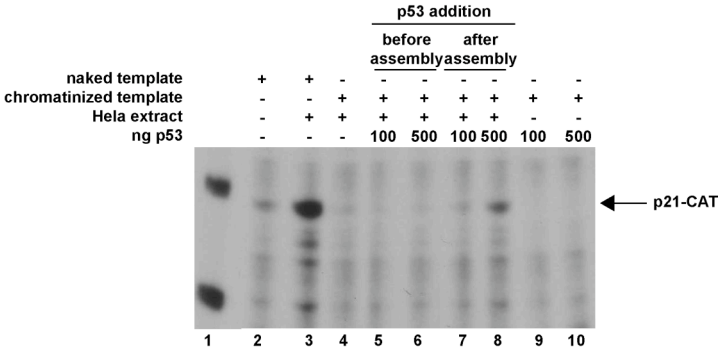
The indicated luciferase reporters were cotransfected with either vector or an expression plasmid for human p53 as indicated. . Fold activation was determined compared to the values obtained for each reporter in the presence of vector. Data represents the average of three independent experiments performed in duplicate. Error bars represent standard deviation.

As was noted, the planned approach to ablate expression of candidate GC box binding proteins may be problematic if there is redundancy among the proteins in our list of likely candidates. We therefore have focused in a biochemical approach to identify this factor. Although it was proposed to perform DNA affinity chromatography, it became apparent that such an approach would likely identify the same proteins that were being screened in the siRNA approach and hence their validation would suffer from the same drawbacks. With this in mind, an in vitro transcription system has been established using chromatinized templates. Identification of a cooperating factor using a functional read-out is expected to be far more useful than relying solely on DNA binding to the GC box. Thus, instead of merely identifying GC box-binding proteins, it should be possible to purify factors that cooperate with p53 to induce transcriptional repression. Through the use of the appropriate reporters, it also will be possible to show that this occurs in a manner dependent upon the GC box.

In vitro transcription using chromatinized templates is technically challenging. Thus, a collaboration has begun with Dr. Michele Barton at the M.D. Anderson Cancer Center. Dr. Barton has extensive experience using chromatinized templates for the study of p53-dependent transcription. With her help, an in vitro transcription system has been established which demonstrates transcriptional activation that is strictly dependent upon addition of p53 (Figure 10). The data in Figure 10 demonstrates that a chromatinized template is essential for these studies. The in vitro transcription system was to utilize a reporter containing the isolated site in the minimal promoter construct that is strictly dependent upon addition of p53 and that demonstrates transcriptional repression. Biochemical fractionation of the extract was then to be performed to

identify the factor that is needed for p53-dependent repression. Unfortunately, we were not able to achieve transcriptional repression in vitro.

Figure 10. p53-dependent transcription of the p21 promoter in vitro requires a chromatinized template and is consistent with a role for p53 in chromatin remodeling.
Hela extract was incubated with either naked DNA (lanes 2-3) or a chromatinized template (lanes 4-10), either in the absence (lanes 2-4) or presence (lanes 5-8) of the indicated amounts of purified human p53. Transcription was also performed in the absence of Hela extract (lanes 9-10). Products were determined by primer-extension assay. p53 was added either prior (lanes 5-6) or after (lanes 7-8) chromatin assembly. A product of the correct size is only detected when p53 is added after assembly of chromatin. Lane 1 contains molecular weight markers.



Key Research Accomplishments

- Demonstrated that Cdc25B but not Cdc25C inhibits cellular proliferation in a manner dependent upon p53
- Showed that the ability of Cdc25B to inhibit cell proliferation involves activation of DNA damage checkpoints
- Showed that overexpression of Cdc25C sensitizes breast cells to treatment with chemotherapeutic agents which induce DNA damage
- Showed that DNA damage induces downregulation of Cdc25C protein and RNA in breast cancer cells
- Demonstrated that Cdc25B is overexpressed in a majority of human breast carcinoma cell lines
- Showed that Cdc25C levels in a panel of human breast carcinoma cell lines correlates with p53 status
- Demonstrated that Cdc25B, but not Cdc25C is required for cell proliferation
- Demonstrated that Sp1 or Sp3 do not contribute to p53-dependent transcriptional repression of Cdc25C
- Determined the minimal element necessary for p53-dependent repression of Cdc25C

Reportable Outcomes

- Established expression constructs for hybrid Cdc25B/Cdc25C proteins and showed that they express the appropriately
- Established an in vitro transcription system which is p53-dependent and utilizes a chromatinized template
- Established methods for siRNA-mediated ablation of Cdc25B and Cdc25C
- Generated expression plasmids for Cdc25C
- Established methods for siRNA-mediated ablation of Sp1 and Sp3
- Varmeh-Ziaie, S. and Manfredi, J.J. 2007. The dual-specificity phosphatase Cdc25B, but not the closely related Cdc25C, is capable of inhibiting cellular proliferation in a manner dependent upon its catalytic activity. J Biol Chem 282:24633-24641. (http://www.jbc.org/cgi/content/abstract/282/34/24633)
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Conclusions

Although the notion that Cdc25B plays a role in human cancer is not new, the idea that Cdc25C may also be important is an intriguing, novel area of investigation. Further, use of Cdc25C as a tool to design therapeutic strategies to intervene in human breast cancer has been previously unexplored. Much of this specific research is laboratory-based and focuses on feasibility of such approaches. Nevertheless, it represents necessary preliminary studies which will allow further development and translation of these findings in the future with the ultimate goal of establishing a highly effective and targeted therapy for human breast carcinoma.

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Appendices

Varmeh-Ziaie, S. and Manfredi, J.J. 2007. The dual-specificity phosphatase Cdc25B, but not the closely related Cdc25C, is capable of inhibiting cellular proliferation in a manner dependent upon its catalytic activity. J Biol Chem 282:24633-24641. (http://www.jbc.org/cgi/content/abstract/282/34/24633)

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The Dual Specificity Phosphatase Cdc25B, but Not the Closely Related Cdc25C, Is Capable of Inhibiting Cellular Proliferation in a Manner Dependent upon Its Catalytic Activity*

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Cdc25B and Cdc25C are closely related dual specificity phosphatases that activate cyclin-dependent kinases by removal of inhibitory phosphorylations, thereby triggering entry into mitosis. Cdc25B, but not Cdc25C, has been implicated as an oncogene and been shown to be overexpressed in a variety of human tumors. Surprisingly, ectopic expression of Cdc25B, but not Cdc25C, inhibits cell proliferation in long term assays. Chimeric proteins generated from the two phosphatases show that the anti-proliferative activity is associated with the C-terminal end of Cdc25B. Indeed, the catalytic domain of Cdc25B is sufficient to suppress cell viability in a manner partially dependent upon its C-terminal 26 amino acids that is shown to influence substrate binding. Mutation analysis demonstrates that both the phosphatase activity of Cdc25B as well as its ability to interact with its substrates contribute to the inhibition of cell proliferation. These results demonstrate key differences in the biological activities of Cdc25B and Cdc25C caused by differential substrate affinity and recognition. This also argues that the antiproliferative activity of Cdc25B needs to be overcome for it to act as an oncogene during tumorigenesis.

In mammalian cells, cyclin-dependent kinase 1 (Cdk1)[3] activity regulates entry into mitosis, whereas Cdk2 action primarily determines initiation of the cell cycle and proper progression through the S phase (1). Cdk1 and Cdk2 activities are regulated at several levels. These include binding to different cyclins, activation by phosphorylation of a regulatory threonine (threonine 161 in Cdk1 or threonine 160 in Cdk2), reversible phosphorylation at two inhibitory residues (threonine 14 and tyrosine 15), subcellular localization, and binding to cyclin-dependent kinase inhibitors (1). The last step in activation of Cdk1 and Cdk2 is removal of the inhibitory phosphates from threonine 14 and tyrosine 15. In mammalian cells, this dephosphorylation event is accomplished by three related dual specificity phosphatases: Cdc25A, Cdc25B, and Cdc25C (2–6). Cdc25A is involved in the initiation of DNA replication as well as mitosis by targeting Cdk2 and Cdk1, respectively (7). In contrast, Cdc25B and Cdc25C induce mitosis by activating Cdk1/cyclin B (7, 8). Cdc25B has been implicated as the initiating phosphatase (9, 10). Activated Cdk1/cyclin B then phosphorylates and activates Cdc25C, which in turn keeps Cdk1/cyclin B active, creating a positive feedback loop that drives the cell through mitosis (11). Cdc25B can dephosphorylate $pT^{14}pY^{15}$Cdk2/cyclin A *in vitro* (12–15). A role for Cdc25B in activation of the G_2 pool of Cdk2/cyclin A has also been suggested (16).

The functions of Cdc25B and Cdc25C are regulated at transcriptional and post-translational levels (7). Modification by phosphorylation and dephosphorylation has been shown to determine their protein stability, activity, substrate specificity, interaction with regulatory proteins, and subcellular localization (7, 9, 14, 17–24). Cdc25B is a relatively unstable protein, which is detected from S phase until the beginning of mitosis (25–27). Its phosphorylation by Cdk1/cyclin A targets it for degradation by the proteasome (17). Cdc25C levels, on the other hand, do not fluctuate during the cell cycle (7). Phosphorylation of Cdc25B has been suggested to be cell cycle-dependent and a determinant for its substrate specificity (9, 14). Cdc25B immunoprecipitated from cells in S phase is active toward $pT^{14}pY^{15}$Cdk2/cyclin A, whereas it targets $pT^{14}pY^{15}$Cdk1/cyclin B when immunoprecipitated from extracts of cells in the G_2/M phases. Abrupt hyperphosphorylation of Cdc25C at the G_2/M transition is believed to activate phosphatase activity toward its substrate $pT^{14}pY^{15}$Cdk1/cyclin B (11). Phosphorylation on specific residues also transforms these phosphatases into targets for certain members of the 14-3-3 protein family. This in turn regulates their activities and subcellular localization under normal conditions or in response to activation of cell cycle checkpoints (19–23, 28–31). Subcellular localization of Cdc25B and Cdc25C changes during the cell cycle and determines accessibility to their substrates (7, 9, 18, 22, 25, 29–32).

Cdc25B and Cdc25C are of comparable size (~500 amino acids). Their N termini, consisting of approximately 300 residues, are believed to have regulatory functions and show a low degree of sequence homology (20% identity) (7). These regions contain nuclear import and export signals and binding sites for 14-3-3 family members. The majority of the regulatory phosphorylations occur within the N termini of Cdc25B and Cdc25C (11, 14). Their C termini (~200 amino acids in length)

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; kd, kilodalton.

contain the catalytic domain (starting with a conserved LIDG motif and including a HCX_5R motif) and are quite similar (61% identity).

Several studies have investigated the roles of the N-terminal regulatory domains and C-terminal catalytic regions in the activities of Cdc25B and Cdc25C *in vitro*. Here chimeric Cdc25B and Cdc25C proteins are used in a cellular context to determine the roles of different regions of Cdc25B and Cdc25C in regulation of cellular proliferation as well as substrate recognition. Moreover, it is demonstrated that overexpression of Cdc25B, but not Cdc25C, inhibits cell proliferation. It is shown that the catalytic activity of Cdc25B as well as its interaction with Cdks is important for its ability to suppress cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture—Human HCT116 (colon carcinoma) and U2OS (osteosarcoma) cells were grown as monolayers in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 10% penicillin/streptomycin (Invitrogen) at 5% CO_2 and 37 °C.

Construction of Plasmids and Chimeras—Cdc25B and Cdc25A cDNAs were generated by reverse transcription. Total RNA was extracted from human WI-38 fibroblasts using the RNeasy® mini kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed using the ThermoScript™ RT-PCR system (Invitrogen) also following the manufacturer's instructions. Subsequent PCR amplifications were performed on a Mastercycler Gradient (Eppendorf) in a total volume of 50 μl containing 2 μl of the cDNA reaction, 200 μM dNTPs, 1× PCR buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% TritonX-100 and 1 mg/ml bovine serum albumin), 400 μM of either Cdc25B or Cdc25A-specific primers, and 1 unit of *Pfu* Turbo DNA polymerase (Stratagene). The following primers were used for PCR amplification. For Cdc25B, the primers were 5′-ATG GAG GTG CCC CAG C (forward) and 5′-GCG CGA TAT CTT ATC ACT GGT CCT GCA GCC G (reverse). For Cdc25A, the primers were 5′-ATG GAA CTG GGC CCG GA (forward) and 5′-GCG CGA TAT CTT TCA GAG CTT CTT CAG ACG (reverse). For Cdc25C, the primers were 5′-ATG TCT ACG GAA CTC TTC (forward) and 5′-GCG CGA TAT CTC ATG GGC TCA TGT C (reverse). Cdc25C was amplified from a plasmid. PCR conditions were as follow: a hot start at 94 °C for 2 min was followed by (for Cdc25B and Cdc25A) 30 cycles of the cycling step (94 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min) or for Cdc25C, 18 cycles of the cycling step (94 °C for 25 s, 61 °C for 20 s, 68 °C for 30 s) and an additional extension time of 10 min at 68 °C.

To generate the 25B/C and 25C/B chimeras, sequences corresponding to the N and C termini of Cdc25B and Cdc25C were PCR-amplified. The following primer sets were used. The N-terminal part of Cdc25B was amplified with 5′-ATGGAGGTGCCCCAGC (forward) and 5′-TCGGTGGTCACTGTCC (reverse). The C terminus of Cdc25B was amplified with 5′-GAGCTGATTGGAGATTAC (forward) and 5′-GCGCGATATCTTATCACTGGTCCTGCAGCCG (reverse). The N terminus of Cdc25C was amplified with 5′-ATGTCTACGGAACTCTTC (forward) and reverse 5′-CCCCTGGTTAGAATC (reverse). The C terminus of Cdc25C was amplified with 5′-CACCTGATTGGTGATTTTTC (forward) and 5′-GCGCGATATCTTATCATGGGCTCATGTC (reverse). Underlining indicates the restriction sites for EcoRV. These fragments were digested with BamHI and EcoRV, purified, and blunt end-ligated (Roche Applied Science). ΔN25B and ΔN25C were generated with the primers used to amplify the C termini of Cdc25B and Cdc25C. ΔN25B/C and ΔN25C/B were produced by fusion PCR. For the first round of PCR, the following primers were used. For the Cdc25B parts, the forward primer used to amplify the C terminus of Cdc25B together with 5′-CTGGCTTCGACACCTCAGTAGCTCATCCTTGAAGGC and the reverse primer used to amplify the C terminus of Cdc25B together with 5′-GCCTTCAAGGATGAGCTACTGAGGTGTCGAAGCCAG were used. For the Cdc25C parts the forward primer used to amplify the C terminus of Cdc25C together with 5′-CTTGAGGCGGAAGGTCTTCAACTCAGTCTTGTGGTC and the reverse primer used to amplify the C terminus of Cdc25C together with 5′-GACCACAAGACTGAGTTGAAGACCTTCCGCCTCAAG were used. Next, the appropriate PCR products were mixed and used as templates for the second round of PCR. The primers used in the second round of PCR were as follows. For ΔN25B/C, they were the forward primer for the C terminus end of Cdc25B and the reverse primer for the C-terminal end of Cdc25C. For ΔN25C/B, they were the forward primer for the C-terminal end of Cdc25C and the reverse primer for the C terminus of Cdc25B. The sequence encoding the FLAG epitope contained a restriction site for BamHI (underlined) (GCGC GGA TCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG) and was contained within the 5′ end of each forward primer. A restriction site for EcoRV (underlined) was included at the beginning of some of the reverse primers. The PCR products were cleaved with BamHI and EcoRV, purified, and subcloned into the pcDNA3 plasmid. The identity of the plasmids was confirmed by DNA sequencing.

Site-directed Mutagenesis—The catalytically inactive mutants of full-length Cdc25B (C488S) and Cdc25C (C377S) and their derivatives were generated using the QuikChange site-directed mutagenesis kit (Stratagene) following protocols provided by the manufacturer. Plasmids carrying the FLAG-tagged full-length Cdc25B, Cdc25C, or their derivatives were used as templates in the PCRs. The following primer sets were used. For mutant Cdc25B, it was 5′-ATT TTC CAC TCT GAA TTC TCA TCT GAG CGT GGG CCC (forward) and GGG CCC ACG CTC AGA TGA GAA TTC AGA GTG GAA AAT (reverse). For mutant Cdc25C, it was 5′-GTG TTC CAC TCT GAA TTC TCC TCA GAG AGG GGC CCC (forward) and 5′-GGG GCC CCT CTC TGA GGA GAA TTC AGA GTG GAA CAC (reverse). For Cdc25B416, it was 5′-GAC CTC AAG TAC ATC GCA CCA GAA ACG ATG GTG (forward) and 5′-CAC CAT CGT TTC TGG TGC GAT GTA CTT GAG GTC (reverse). For Cdc25B470, it was 5′-AGC TTC CTA CTG AAG GCC CCC ATC GCG CCC TGT (forward) and 5′-ACA GGG CGC GAT GGG GGC CTT CAG TAG GAA GCT (reverse). The mutated bases are underlined.

Immunoblotting—The cells were lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 mM Na_3VO_4, 10 mM NaF, 80 mM β-glycerophosphate, 1 mM

phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, 5 mg/ml leupeptin and pepstatin. The lysates were clarified by centrifugation (10,000 × *g* for 10 min at 4 °C) and immunoblotted. The following antibodies were used: mouse anti-FLAG was purchased from Sigma. Rabbit anti-Cdc25B (H-85), mouse anti-Cdc25A (F-6), mouse anti-Cdc25C (H-6), rabbit anti-Cdk2 (M2), mouse anti-Cdk1 (C-19), rabbit anti- $pT^{14}pY^{15}$Cdk1 (cross-reacts with Cdk2), rabbit anti-cyclin B (H-433), and rabbit anti-cyclin A (H-432) were from Santa Cruz Biotechnology. Rabbit anti-Tyr(P)^{15} Cdk1 (cross-reacts with Cdk2) was from Cell Signaling Technology. Antibody to mouse anti-β-actin was from Oncogene Research Products. Peroxidase-conjugated goat antibodies against rabbit or mouse IGG (MP Biomedicals) were used as secondary antibodies. The signals were detected using ECL reagents (Amersham Biosciences) and autoradiography films (Labscientific, Inc.).

Colony Formation Assays—Each of the plasmids expressing Cdc25B, Cdc25C, Cdc25A, mutant Cdc25B, mutant Cdc25C, their derivatives, or empty vector was co-transfected with a plasmid conferring puromycin resistance into exponentially growing HCT116 cells using Lipofectamine™ reagent and Plus reagent (Invitrogen) following the manufacturer's instructions. Forty-eight hours post-transfection, the cells were subjected to puromycin (2 μg/ml) selection for 12–14 days. The colonies were fixed and stained with Giemsa (Sigma-Aldrich).

Immunoprecipitations—HCT116 cells were transfected as described. The cells were lysed in the buffer described for immunoblotting. Mouse anti-FLAG® M2 affinity gel (Sigma) was used in immunoprecipitation assays, which were performed following manufacturer's protocols. The immunoprecipitates were then immunoblotted as described.

Recombinant Adenoviruses—The FLAG-tagged open reading frames of Cdc25B, mutant Cdc25B, and Cdc25C were cut out of plasmids and subcloned into the pAdTrack-CMV shuttle vectors. These constructs were linearized by digestion with PmeI and were co-transformed together with the adenoviral backbone plasmid pAdEasy-1 into the homologous recombination-competent *Escherichia coli* strain BJ5183. The resultant recombinants were linearized with PacI and transfected into the adenovirus packaging cell line, 293T. High titer of recombinant adenoviruses was obtained by several rounds of infection. The cells were infected with multiplicity of infection of 8–12 for Ad-25B, 5–10 for Ad-25C, and 35–40 for Ad-mt25B.

RESULTS

Cdc25B but Not Cdc25C Inhibits Colony Formation—The long term consequences of overexpression of Cdc25B and Cdc25C on cell proliferation were investigated. The open reading frames of Cdc25B and Cdc25A were amplified from RNA extracted from WI-38 human fibroblasts using reverse transcription-PCR. The open reading frames of all three phosphatases were FLAG-tagged at their N termini and subcloned into the pcDNA3 vector (see "Experimental Procedures"). Each of these constructs or empty vector was co-transfected together with a plasmid conferring puromycin resistance into HCT116 cells. Immunoblotting using transiently transfected cell lysates and anti-FLAG antibody or specific antibody to each protein showed that these proteins were expressed at similar levels and

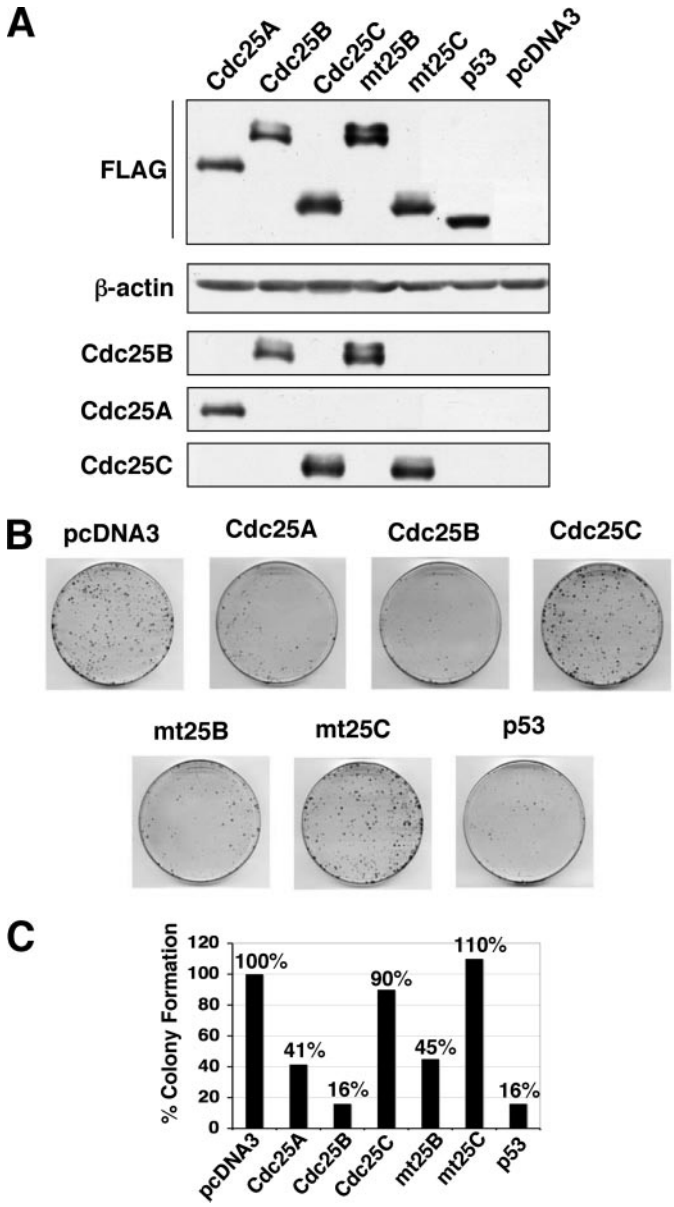

FIGURE 1. **Ectopic expression of Cdc25B but not Cdc25C inhibits proliferation in long term assays.** pcDNA3 vectors expressing indicated FLAG-tagged proteins or empty vector were co-transfected with a puromycin-resistant plasmid into HCT116 cells. *A*, cells were lysed 24 h post-transfection, and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody (*upper panel*) and antibodies specific to each protein as indicated (*lower panels*). β-Actin is a loading control. *B*, cells were subjected to puromycin selection for 12–14 days followed by staining with Giemsa. Representative plates are shown. *C*, bar graph shows the numbers of colonies formed in the presence of each construct as percentages of that of empty vector. The numbers of colonies in the presence of vector is set at 100%. Cdc25A and p53 were included as controls. The average values of two experiments performed in duplicate are shown.

exhibited the expected mobility (65 kd for Cdc25B, 55 kd for Cdc25C, and 60 kd for Cdc25A) (Fig. 1*A*). These constructs were then used in colony formation assays. Forty-eight hours post-transfection, HCT116 cells were subjected to puromycin selection for 12–14 days before fixing and staining the colonies with Giemsa (Fig. 1*B*). The tumor suppressor protein p53 was included in this experiment as a control. Consistent with previous published observations, p53 expression inhibited colony formation (33). Cdc25A was included in this experiment as a

control for overexpression of an oncoprotein and appeared to impose a negative effect on cell proliferation (41%) but was not as effective as Cdc25B. Cdc25B significantly reduced the number of colonies (16%) compared with empty vector (set at 100%) (Fig. 1, *B* and *C*). By contrast, Cdc25C did not have any significant effect on the number, size, or shape of the colonies (90 and 110%, respectively) (Fig. 1, *B* and *C*). These results indicate that ectopic expression of Cdc25B but not Cdc25C reduces long term viability of the cells.

The Phosphatase Activity of Cdc25B Contributes to the Ability to Inhibit Cell Proliferation—It is well established that mutating the catalytic cysteine abolishes the phosphatase activities of the Cdc25s (6, 15, 34). To verify the role of the phosphatase activities of Cdc25B and Cdc25C in these assays, their catalytically inactive mutants Cdc25B (C488S) and Cdc25C (C377S) (referred to as mt25B and mt25C) were generated and were FLAG-tagged at their N termini. When compared with wild type Cdc25B (16%), the ability to suppress colony formation was impaired with the mutant Cdc25B (45%) (Fig. 1, *B* and *C*). This result argues that the phosphatase activity of Cdc25B accounts for a significant part of its antiproliferative ability. Nevertheless the mutant Cdc25B retained some ability to suppress colony formation. This is most likely due to its ability to sequester its substrates and deprive the cell of their functions (6, 15, 34). By contrast, neither the inactive form of Cdc25C nor the wild type protein had any significant effect on the number, size, or shape of the colonies (90 and 110%, respectively) (Fig. 1, *B* and *C*). Thus, both the active and phosphatase-dead forms of Cdc25B and Cdc25C differ in their biological effects. Importantly, the phosphatase activity of Cdc25B dramatically enhances its ability to suppress cell growth.

The Catalytic Domain of Cdc25B Is Sufficient to Suppress Cell Viability—The N-terminal ends of Cdc25B and Cdc25C have been implicated in their regulation (14). To elucidate the role of the N terminus of Cdc25B in its antiproliferative activity, chimeras in which the N termini of Cdc25B and Cdc25C were exchanged were generated (25B/C and 25C/B) (Fig. 2). 25B/C consists of residues 1–390 of Cdc25B fused to residues 281–473 of Cdc25C (Fig. 2). The N terminus of 25C/B encompasses residues 1–280 of Cdc25C, and it was fused to residues 391–580 of Cdc25B (Fig. 2). The chimeras were generated by blunt end cloning to avoid possible influence of irrelevant sequences. As controls, constructs expressing only the catalytic domains of Cdc25B (ΔN25B) and Cdc25C (ΔN25C) and their corresponding catalytically inactive mutants were generated (Fig. 2). ΔN25B contains residues 391–580 of Cdc25B and ΔN25C residues 281–473 of Cdc25C. All of the constructs were FLAG-tagged at the N terminus. Each of these constructs or empty vectors was co-transfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours post-transfection, the cell samples were split in half. One half were lysed and immunoblotted with FLAG antibody to verify similar expression levels and size of the proteins (Fig. 3, *A* and *B*). To further validate these constructs, the immunoblots were then stripped and subsequently probed with specific antibodies against the N or C terminus of Cdc25B or Cdc25C (Fig. 3, *A* and *B*). The second half of each cell sample was subjected to colony formation assays. The number of colonies formed by the 25B/C construct

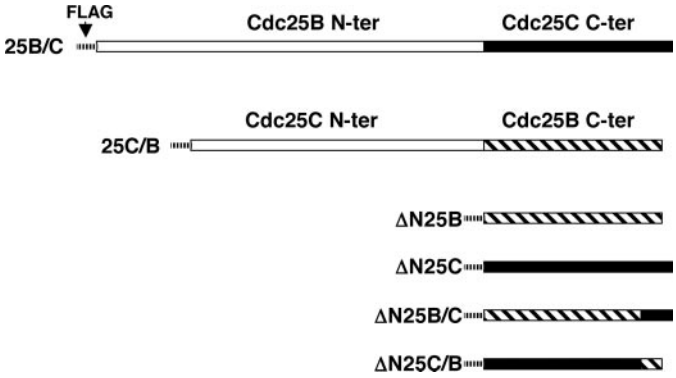

FIGURE 2. **Expression constructs for FLAG-tagged chimeras of Cdc25B and Cdc25C and their isolated C-terminal regions were generated.** Sequences corresponding to the N- and C-terminal ends of Cdc25B and Cdc25C were PCR-amplified. The N-terminal end of Cdc25B encompasses residues 1–390, and that of Cdc25C encompasses residues 1–280. The C-terminal end of Cdc25B (*hatched*) and Cdc25C (*black*) consists of residues 391–580 and residues 281–473, respectively. The constructs expressing 25B/C and 25C/B were generated by blunt end ligation of the indicated parts of the proteins. ΔN25B and ΔN25C express the C-terminal ends of Cdc25B and Cdc25C, respectively. The constructs expressing ΔN25B/C and ΔN25C/B were generated by fusion PCR. ΔN25B/C expresses residues 391–554 of Cdc25B fused to residues 445–473 of Cdc25C. ΔN25C/B expresses residues 281–444 of Cdc25C fused to residues 555–580 of Cdc25B. All of the constructs were FLAG-tagged at their N-terminal ends. The position of the FLAG epitope is indicated.

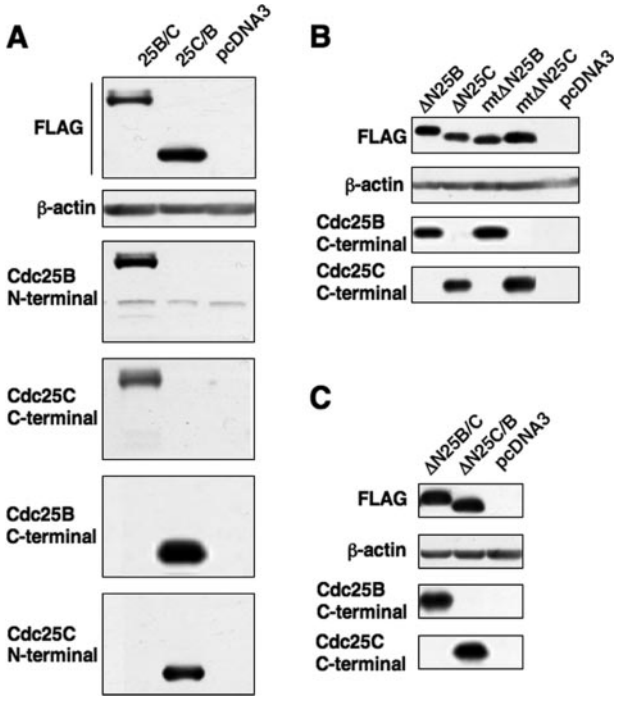

FIGURE 3. **Various Cdc25B and Cdc25C proteins are expressed at comparable levels.** pcDNA3 vectors expressing the indicated FLAG-tagged proteins or empty vector were transfected into HCT116 cells. Twenty-four hours later, the cells were lysed, and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody or specific antibodies against the N or C terminus of Cdc25B or Cdc25C. β-Actin is loading control. *A*, 25B/C and 25C/B chimeras. *B* and *C*, ΔN25B and ΔN25C (*B*) and their corresponding catalytically inactive mutants ΔN25B/C and ΔN25C/B (*C*).

was 60% of that of empty vector (set at 100%) (Fig. 4, *A* and *B*). This was 4-fold greater than that of full-length Cdc25B (16%), suggesting that the N-terminal region of Cdc25B plays a role in its antiproliferative activity. However, the result with 25B/C was still 1.5-fold less than full-length Cdc25C (90%), arguing

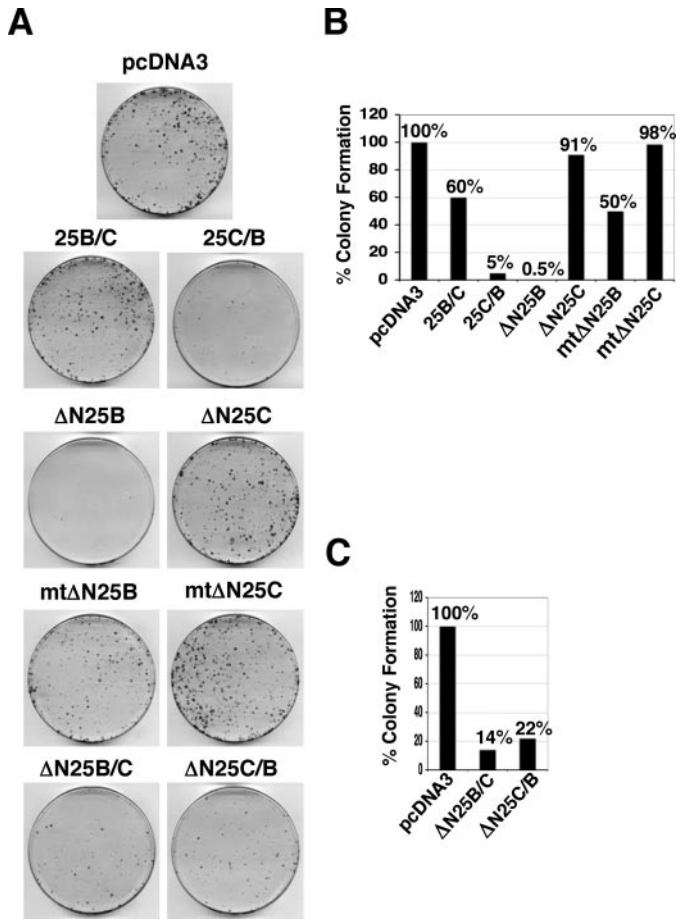

FIGURE 4. **The C-terminal end of Cdc25B is sufficient to suppress cell viability.** *A*, indicated constructs or empty vector were co-transfected with a puromycin-resistant plasmid into HCT116 cells. Transfected cells were selected by feeding the cells with medium containing puromycin (2 μg/ml) for 12–14 days. The colonies were fixed and stained with Giemsa. Representative plates are shown. *B* and *C*, bar graphs show the numbers of colonies formed in the presence of each construct as the percentages of that formed with empty vector (set at 100%). The average values of two experiments performed in duplicate are shown.

that the N-terminal part of Cdc25C may negatively regulate the activity. The 25C/B chimera and ΔN25B on the other hand appeared to be more efficient than the full-length Cdc25B in inhibiting cell proliferation because the number of colonies formed following their overexpression were 5 and 0.5% compared with the empty vector (Fig. 4, *A* and *B*). These results indicate that the C terminus of Cdc25B is responsible for its ability to inhibit cell proliferation, and the N terminus of Cdc25B when fused to its C terminus negatively regulates this activity. The mtΔN25B reduced the number of colonies (50%), but not as efficiently as the wild type (0.5%), further confirming that the catalytic activity of Cdc25B plays a major role in its ability to suppress cell viability. The ΔN25C and its mutant behaved like the full-length Cdc25C and did not affect colony formation significantly (Fig. 4, *A* and *B*). These results also suggest that the N terminus of Cdc25B may also be involved in the suppression of colony formation that resulted from overexpression of 25B/C.

Given the high homology (61% identity) between the C-terminal domains of Cdc25B and Cdc25C, the effect of their overexpression on colony formation was surprising. The C termini of Cdc25B and Cdc25C, however, show much less homology at the extreme C-terminal end (24% identity) (the last 26 residues of Cdc25B and last 29 residues of Cdc25C). Biochemical studies have suggested that the two arginines, Arg^{556} and Arg^{562}, within the last 17 residues (referred to as a "docking" region) of Cdc25B1 are important for its recognition and specific interaction with $pT^{14}pY^{15}$Cdk2/cyclin A (12, 13). To investigate the effect of this putative docking region on the ability of the C terminus of Cdc25B to inhibit cell proliferation, the C-terminal tails of ΔN25B and ΔN25C were exchanged. Constructs were generated by using fusion PCR so that there was no foreign sequence introduced as a linker. These constructs are referred to as ΔNB/C and ΔNC/B (Fig. 2). ΔNB/C consists of residues 391–554 of Cdc25B fused to residues 445–473 of Cdc25C (Fig. 2). ΔNC/B encompasses residues 281–444 of Cdc25C fused to residues 555–580 of Cdc25B (Fig. 2). They were validated by sequencing of constructs and immunoblotting of expressed proteins using either FLAG or specific antibodies against the C termini of Cdc25B or Cdc25C (Fig. 3*C*). These constructs were used in colony formation assays. Interestingly both ΔN25B/C and ΔN25C/B appeared to be able to suppress cell proliferation when overexpressed (Fig. 4, *A* and *C*). The number of colonies formed following overexpression of ΔN25B/C was 14% of that of empty vector. This is 28-fold greater than the number of colonies formed by ΔN25B (0.5%), suggesting a role for the last 26 amino acids of Cdc25B in its antiproliferative ability. The difference between the number of colonies formed by ΔN25C and ΔN25C/B was striking (91 and 22%, respectively) (Figs. 4, *A* and *B*, and 1, *B* and *C*). These experiments suggest that either the last 26 residues of Cdc25B conferred antiproliferative ability to the catalytic domain of Cdc25C or the last 29 amino acids of Cdc25C negatively regulate its activity.

The N-terminal Portion of Cdc25B Is Dispensable for Its Affinity and Recognition of Its Known Substrates, Whereas Its Last 26 Residues Play a Role in Its Substrate Specificity—Ectopic expression of the C terminus of Cdc25B alone or fused to the N terminus of Cdc25C was more efficient in suppressing cell viability, whereas overexpression of its mutant was not (Fig. 4). This suggests that the C terminus of Cdc25B independently of its N-terminal end is capable of binding and dephosphorylating its substrates. Overexpression of Cdc25C or its derivatives, on the other hand, did not affect colony formation, which would be consistent with a lower affinity for its substrates. To examine this possibility, immunoprecipitation assays were performed. Each of the above constructs or their corresponding inactive mutants or empty vector were transfected into HCT116 cells. The expressed proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were first immunoblotted for FLAG to verify expression and similar loading (Fig. 5). The presence of the known substrates for Cdc25B and Cdc25C in the immunoprecipitates was investigated. As shown in Fig. 5, mutant Cdc25B, mt25C/B and mtΔN25B formed stable complexes with equal amounts of $pT^{14}pY^{15}$Cdk1 and $pT^{14}pY^{15}$Cdk2 (top band of Cdk1 and bottom band of Cdk2) and pY^{15}-Cdk1 (middle band), cyclin B, and cyclin A. Cdk1 is detected as three bands on a SDS-polyacrylamide gel (34). The middle band has been suggested to be phosphorylated only on tyrosine 15 (35). The bottom band is fully dephosphorylated on

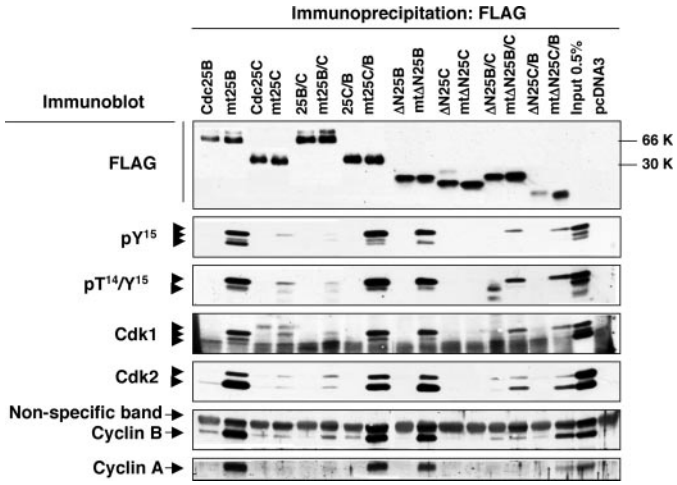

FIGURE 5. **The C-terminal 26 residues of Cdc25B but not its N-terminal region are important for recognition of its substrates.** Constructs expressing the indicated proteins, their catalytically inactive mutants, or empty vector were transfected into HCT116 cells. Twenty-four hours later the cell lysates were prepared, and the proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were immunoblotted to detect the FLAG epitope as well as with specific antibodies against the indicated cell cycle regulators.

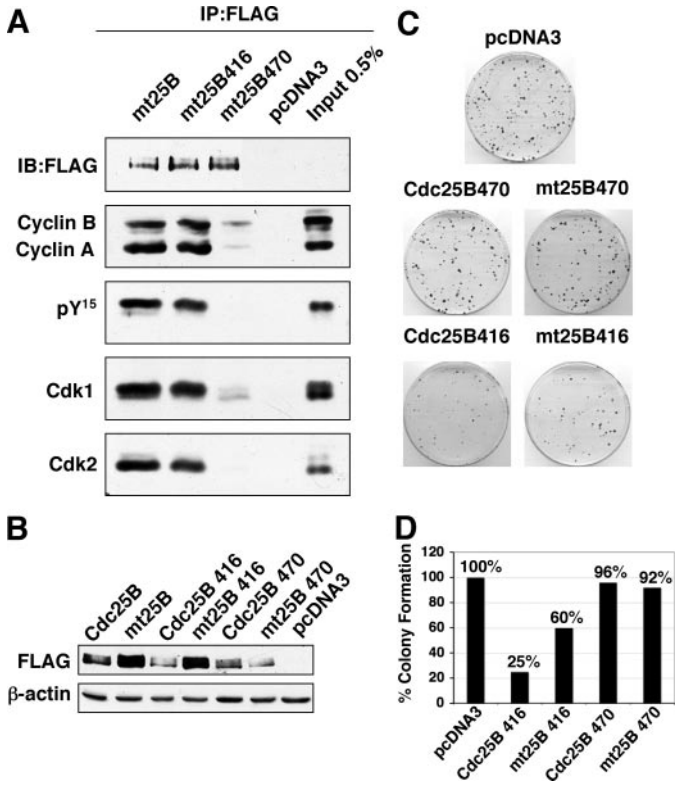

FIGURE 6. **Integrity of serine 470 is essential for interaction of Cdc25B with its substrates and its antiproliferative effect.** *A*, the catalytically inactive mutants of either wild type Cdc25B, Cdc25B416, Cdc25B470, or empty vector were transfected into HCT116 cells. Twenty-four hours following transfection the cells were lysed, and the proteins were immunoprecipitated (*IP*) using anti-FLAG antibody. The immunoprecipitates were immunoblotted with the indicated antibodies. *B*, the indicated constructs were co-transfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours later, the cell lysates from these cells were subjected to immunoblot analyses to verify similar expression levels of the proteins using anti-FLAG antibody. β-Actin is a loading control. *C*, alternatively, the cells were subjected to puromycin selection for 2 weeks, and the colonies were stained with Giemsa. Representative plates are shown. *D*, bar graph shows the numbers of colonies formed in the presence of each construct as a percentage of that of empty vector. The numbers of colonies in the presence of vector is set at 100%. The average values of three experiments performed in duplicate are shown.

these residues. Cdk2 appears as two bands on a SDS-polyacrylamide gel. The bottom band represents $pT^{14}pY^{15}$Cdk2 (36). Lack of the N terminus of Cdc25B or fusion of the N terminus of Cdc25C to the C terminus of Cdc25B did not significantly affect the affinity of the mutants for these cell cycle regulators (Fig. 5). Compared with these mutants, mtΔN25B/C, however, had a significantly reduced affinity for $pT^{14}pY^{15}$Cdk1/cyclin B and $pT^{14}pY^{15}$Cdk2/cyclin A (Fig. 5). Wild type ΔN25B/C was found to bind to the dephosphorylated and $Tyr(P)^{15}$ forms of Cdk1 (Fig. 5). Interestingly, mtΔNC/B was able to form a complex with all of these, although it bound to a lesser extent as compared with ΔN25B (Fig. 5). These results indicate that either the last 26 residues of Cdc25B conferred an increase in affinity of the catalytic domain of Cdc25C for $pT^{14}pY^{15}$Cdk1/cyclin B and $pT^{14}pY^{15}$Cdk2/cyclin A or the last 29 residues inhibit the interaction between the catalytic domain of Cdc25C and these complexes. Mutant Cdc25C, mt25B/C, and mtΔN25C, on the other hand, showed lower affinities for these cell cycle regulators, as evidenced by the barely detectable amounts seen by immunoblotting (Fig. 5). As expected, none of the examined cell cycle regulators were found in immunoprecipitates from cells expressing the wild type forms of the above constructs (Fig. 5).

Serine 470 Is Essential for Interaction between Cdc25B and Its Substrates and Its Antiproliferative Activity—Cdc25B contains several serines that are followed by prolines, and as such they are candidates for phosphorylation by the Cdks (37). It was hypothesized that phosphorylation of at least one of these serines would enhance the interaction between Cdc25B and its substrates. Because the C terminus of Cdc25B behaved similarly to the full-length protein in colony formation and immunoprecipitation assays, the two serines at positions 416 and 470 were mutated to alanines in both wild type and catalytically inactive Cdc25B. Wild type Cdc25B mutated at either position will be referred to as Cdc25B416 and Cdc25B470, respectively. Catalytically inactive Cdc25B mutated at these residues will be referred to as mt25B416 and mt25B470. First, these mutants were used in immunoprecipitation assays. Interestingly, mt25B470 was incapable of forming complexes with either Cdk1 or Cdk2 and their cyclin partners (cyclins B and A) (Fig. 6*A*). The affinity of mt25B416 for its substrates, on the other hand, was only slightly reduced compared with mtCdc25B (Fig. 6*A*). To investigate how the inability of Cdc25B470 to bind to its substrates affects its antiproliferative property, these new constructs were used in colony formation assays. First, immunoblot analyses were performed to ensure similar expression levels from all the constructs (Fig. 6*B*). Overexpression of Cdc25B416, similar to wild type Cdc25B, was toxic to cells (25%), whereas that of Cdc25B470 or its corresponding catalytic mutant did not affect cell proliferation (96 and 92%, respectively) (Fig. 6, *C* and *D*). The ability of mt25B416 to suppress colony formation was similar to mtCdc25B (60%) (Fig. 6, *C* and *D*). Two important conclusions can be drawn from these results. First, serine 470 of Cdc25B is essential for the interaction with substrates. Second, the antiproliferative effect of Cdc25B that involves its phosphatase activity also is dependent

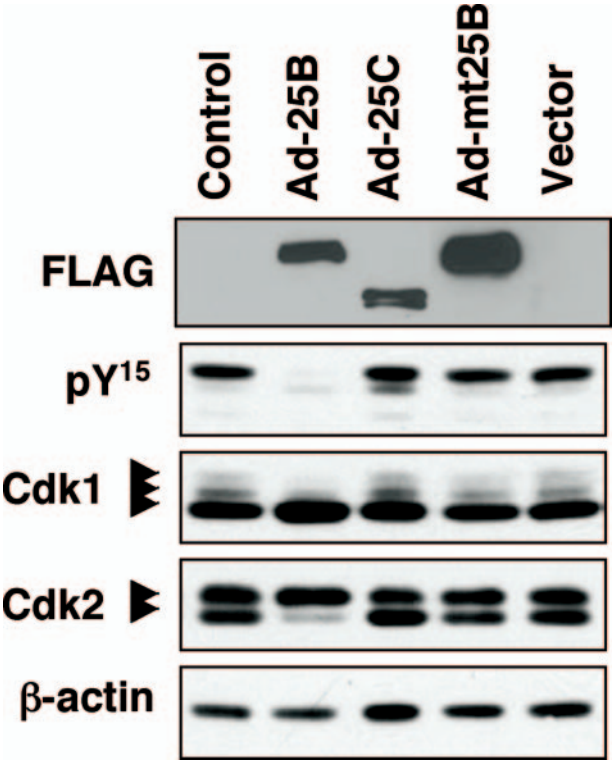

FIGURE 7. **Elevated levels of Cdc25B but not Cdc25C causes dephosphorylation of Cdk1 and Cdk2 on tyrosine 15.** U2OS cells were infected with adenoviruses expressing the indicated FLAG-tagged proteins or empty vector. The cells were lysed 24 h later and immunoblotted using the shown antibodies. β-Actin is used as a loading control.

upon binding to Cdks. This supports the notion that the ability of Cdc25B to inhibit cell growth involves activation of Cdks.

Dephosphorylation of Tyrosine 15 on Cdk1 and Cdk 2 Occurs in Cells Undergoing Cdc25B-induced Growth Arrest—Cdc25B and Cdc25C are known to trigger mitosis by dephosphorylating Cdk1 on threonine 14 and tyrosine 15 (2–6). To more directly demonstrate that the antiproliferative ability of Cdc25B involves activation of the CDKs, recombinant adenoviruses expressing Cdc25B (Ad-25B), its catalytic inactive mutant (Ad-mt25B) and Cdc25C (Ad-25C) were constructed. This approach was chosen because establishing stable cell lines overexpressing Cdc25B is not feasible (Fig. 1*B*), and conventional transient transfection approaches target only a small percentage of the cells. U2OS cells were infected with each virus and then assayed 24 h later. The ability of Cdc25B but not Cdc25C to induce growth arrest was confirmed by examining bromodeoxyuridine incorporation (data not shown). Phosphorylation of Cdk1 and Cdk2 on tyrosine 15 was then analyzed by immunoblotting (Fig. 7). Expression of Cdc25B caused dephosphorylation of both CDKs on tyrosine 15, which is necessary for their activation as kinases. Expression of mutant Cdc25B, despite being expressed at considerably higher levels than the wild type Cdc25B, did not result in dephosphorylation of Cdk1 and Cdk2 on this residue (Fig. 7). Cdc25C has a lower affinity for binding to its substrates (Fig. 5). Consistent with this, expression of Cdc25C at similar levels as Cdc25B did not result in dephosphorylation of CDKs on tyrosine 15.

Thus, the ability to inhibit colony formation and induce growth arrest by Cdc25B correlates well with its activity as a tyrosine 15 phosphatase for Cdk1 and Cdk2. Taken together, these results strongly support the notion that Cdc25B inhibits colony formation by a mechanism involving inappropriate activation of its substrates.

DISCUSSION

In this study, biological evidence is provided that confirms biochemical studies comparing activities of Cdc25B and Cdc25C and their chimeras (12–15). It is demonstrated that overexpression of Cdc25B, but not Cdc25C, is detrimental to cell vitality. Furthermore, these data identify serine 470 of Cdc25B as an essential residue for interaction with its substrates, $pT^{14}pY^{15}$Cdk1/cyclin B and $pT^{14}pY^{15}$Cdk2/cyclin A. Finally, it is shown that Cdc25B mutated at this residue is also unable to inhibit cell proliferation. The antiproliferative effect of Cdc25B was not specific to HCT116 cells because similar results were obtained using U2OS (osteosarcoma) and HT1080 (fibrosarcoma) cells (data not shown). The level of Cdc25B expression has been suggested to be a critical regulator of its function (6, 9). Thus, alteration in the level of this phosphatase is expected to influence progression through the cell cycle.

The phosphatase activity of Cdc25B is clearly implicated in its ability to suppress cell proliferation. Although the catalytically inactive form retains some ability to inhibit cell growth, the fully active wild type Cdc25B suppresses colony formation to a substantially greater extent (16% *versus* 45%; Fig. 1, *B* and *C*). This is despite the greater ability of the mutant Cdc25B to interact with its substrates (Fig. 5). Thus, the molecular basis for the antiproliferative activity of wild type Cdc25B appears to be mechanistically distinct from that of the mutant form of the phosphatase and is likely to involve inappropriate catalytic activity toward its Cdk substrates. Although counterintuitive, the notion that proteins with oncogenic potential also exert negative effects on cellular growth is not unprecedented. Indeed, a paradigm has been established in which oncogenes such as Myc or Ras not only induce uncontrolled cell proliferation but also trigger so-called "oncogenic" checkpoints that need to be overcome for full transformation to occur (38). The studies presented here suggest that the Cdc25B oncogene should also be considered an example of this.

Previous studies have demonstrated that overexpression of Cdc25C has no effect on cell cycle progression in short term experiments (9, 19, 39). Colony formation assays in this study demonstrate that exposure to elevated levels of Cdc25C over an extended period of time can also be tolerated by cells. In support of this, stable cell lines derived from 293, U2OS, or HT1080 cells that overexpress Cdc25C were easily established. These lines were viable and did not show any alterations in their cell cycle profiles or morphologies even after numerous passages (Ref. 40 and data not shown). *In vitro* and cellular analyses have suggested that Cdc25C becomes active only after being modified by phosphorylation (9, 14, 39). Thus, the availability or activity of the kinase(s) that phosphorylate Cdc25C could be the limiting factors for its activity.

Removal of the N terminus of Cdc25B enhanced its ability to suppress cell viability (Fig. 4). This is in agreement with previous reports demonstrating that removal of the N terminus of Cdc25B augments its ability to induce mitotic catas-

trophe in cells and increases its activity toward its substrates *in vitro* (14, 22). This outcome may be due to inappropriate subcellular localization because the N terminus of Cdc25B is known to contain the nuclear import and export signals and binding sites for the 14-3-3 family members, which are known to regulate Cdc25B localization and activity (18, 29, 30). The N-terminal region is unlikely to affect the ability of the catalytic domain to bind to its substrates because similar amounts of $pT^{14}pY^{15}$Cdk1/cyclin B and $pT^{14}pY^{15}$Cdk2/cyclin A were found to bind to the mutant forms of full-length or the isolated catalytic domain of Cdc25B in immunoprecipitation assays (Fig. 5).

Similar to Cdc25B, the N-terminal end of Cdc25C contains nuclear import and export signals and binding sites for 14-3-3 proteins (19). In this study, removal of the N terminus of Cdc25C abolished its limited affinity for $pT^{14}pY^{15}$Cdk1/cyclin B and did not affect colony formation (Figs. 4 and 5). Phosphorylation of serine 216 on Cdc25C and subsequent binding to 14-3-3 family members are believed to negatively regulate its activity (19). Thus, removal of the N terminus of Cdc25C would be expected to stimulate its function. On the other hand, hyperphosphorylation of other sites in this region has been associated with its activation (11, 14).

The C-terminal domain of Cdc25B when expressed alone or fused to the N terminus of Cdc25C had a greater ability to suppress colony formation (Fig. 4), suggesting a negative role for the N terminus in regulating Cdc25B activity. This is in agreement with the *in vitro* observation showing that the catalytic activity of the bacterially expressed C-terminal domain of Cdc25B toward *p*-nitrophenyl phosphate, $pT^{14}pY^{15}$Cdk1/cyclin B, or $pT^{14}pY^{15}$Cdk2/cyclin A is greater than the full-length Cdc25B, and fusing the N terminus of Cdc25C to the C terminus end of Cdc25B did not affect this activity (14).

The difference between the consequences of overexpression of the C-terminal of Cdc25B and Cdc25C on colony formation was striking considering their high degree of homology. Exchanging the extreme tails from the C-terminal of Cdc25B and Cdc25C, however, significantly affected their ability for substrate recognition and conferred antiproliferative advantage to the catalytic domain of Cdc25C. Affinity of the C terminus domain of Cdc25B for $pT^{14}pY^{15}$Cdk1/cyclin B was also decreased. The ΔN25C/B chimera, on the other hand, showed an increase affinity for both $pT^{14}pY^{15}$Cdk1/cyclin B and $pT^{14}pY^{15}$Cdk2/cyclin A. Given the biochemical studies proposing the last 17 residues of Cdc25B as a modular "docking" site for $pT^{14}pY^{15}$Cdk2/cyclin A (12, 13), this is likely due to the ability of the last 26 residues of Cdc25B contributing to binding this complex rather than that the last 29 residues of Cdc25C inhibit this interaction. The data presented here suggest that this region is also important for the ability of the C terminus of Cdc25B to bind to $pT^{14}pY^{15}$Cdk1/cyclin B (Fig. 5). Nevertheless, the possibility that the last 29 residues of Cdc25C have a negative effect on its ability to bind to the examined cell cycle regulators cannot be excluded. Biochemical data, however, are not in favor of this possibility because removal of the last 20 residues of the C terminus of Cdc25C did not affect its activity toward $pT^{14}pY^{15}$Cdk2/cyclin A (12).

Ectopic expression of the catalytically inactive forms of Cdc25B or its derivatives inhibited colony formation, although not as efficiently as the wild type forms. Immunoprecipitation assays in this study confirms the previously proposed mechanism by which this mutant may prevent cell cycle progression (6, 15). This mutant was suggested to inhibit or delay entry into mitosis by forming a stable complex with $pT^{14}pY^{15}$Cdk1/cyclin B (6). As seen here, this mutant is also equally capable of binding and sequestering $pT^{14}pY^{15}$Cdk2/cyclin A in cells, suggesting that overexpression of this mutant may affect progression into or through the S phase as well. Thus, survival of 50% of cell population was surprising. One likely explanation is that levels of the inactive mutant were not high enough to compete with the endogenous wild type protein. In support of this is the observation that the inactive Cdc25B, when expressed at the same level as the wild type protein, did not affect cell proliferation. Indeed, it needs to be expressed at significantly higher levels than the wild type protein to achieve a cell cycle arrest with the majority of the cells accumulated at the G_2/M transition (data not shown).

Overexpression of the catalytically inactive Cdc25C has been shown to lead to accumulation of cells in G_2/M. In the colony formation assays performed in this study, however, number, size, or shape of colonies formed by cells transfected with this mutant was comparable with those of the control. One possibility is that the catalytically inactive Cdc25C is less efficient at competing with the endogenous protein as compared with Cdc25B and therefore has to be overexpressed at a considerable higher level. Another possibility is a lack of activating phosphorylations on the mutant Cdc25C. Phosphorylation of Cdc25C has been shown to be required for its activation (11, 14). Consistent with the results of the colony formation assays, mutant Cdc25C showed far less affinity for $pT^{14}pY^{15}$Cdk1/cyclin B relative to mutant Cdc25B in immunoprecipitation assays.

In summary, these results demonstrate key differences in the biological activities of Cdc25B and Cdc25C because of differential substrate affinity and recognition that could explain why Cdc25B, but not Cdc25C, acts as an oncogene. Mutation analysis demonstrates that both the phosphatase activity of Cdc25B as well as its ability to interact with its substrates contributes to the inhibition of cell proliferation. This supports the notion that the ability of Cdc25B to inhibit cell growth is dependent upon its catalytic activity, most likely involving hyperactivation of Cdks and inappropriate cell cycle progression. Importantly, this argues that similar to other transforming genes, the antiproliferative activity of Cdc25B needs to be overcome for it to act as an oncogene during tumorigenesis.

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